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New technologies for studies of the gypsy moth and its pathogens

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■ **ABSTRACT.** The gypsy moth, *Lymantria dispar* L., was introduced into North America in 1868. Within ten years, this forest tree defoliator was in outbreak status due to the preponderance of *Quercus* species that dominated the northeastern forests and are a preferred food. *Lymantria dispar* was introduced without the compliment of natural enemies that occur in European populations, allowing for more frequent and severe outbreaks. In addition, an Asian strain of *L. dispar* has been periodically introduced and presumably eradicated. Unlike the European *L. dispar* strain, the Asian *L. dispar* females, as well as females that are hybrids between the two strains, are able to fly, increasing dispersal capability. The Asian strain is also utilizes more host plants as a food source. During the past 100 years, many strategies for eradication and suppression of European *L. dispar* have been developed and applied. Recent research projects include several molecular approaches, some of which have resulted in important information and usable techniques, some are currently in the developmental stages. These projects have addressed a wide range of research topics, including determination of the genetic basis of virulence of nuclear polyhedrosis virus isolates and examination of a mutant NPV that reduces production in cell culture, and detection of the Asian strain of *L. dispar* and Asian/European hybrids. Another project was initiated to isolate the vitelloginen gene in *L. dispar* in order to examine the role of ecdysone- possibly important in designing biorational *L. dispar* pesticides. A new project is underway to develop a molecular method to identify and distinguish several biotypes of microsporidian (Protozoa) pathogens of *L. dispar.* These research efforts were initiated by the U.S. Forest Service, as well as North American and European institutes, agencies and universities that cooperate in the North American gypsy moth control programs.

■ **RESUMEN. Nuevas tecnologías para el estudio de la polilla gitana y sus patógenos.** La polilla gitana, *Lymantria dispar* L., fue introducida en América del Norte en 1868. Durante diez años este defoliador de árboles de importancia forestal permaneció en estado de explosión demográfica debido a la predominancia de las especies de *Quercus*, que dominan los bosques del nordeste y son su alimento preferido. *Lymantria dispar* fue introducida sin el complemento de los enemigos naturales de las poblaciones europeas, permitiendo más frecuentes y severas explosiones demográficas. Además, una cepa asiática de *L. dispar* ha sido periódicamente introducida y presumiblemente erradicada. En contraposición a la cepa europea, las hembras de la cepa asiática de *L. dispar*, así como las hembras que son híbridos entre las dos cepas, son capaces de volar, incrementando así su capacidad de dispersión. Asimismo, la cepa asiática utiliza más plantas huéspedes como fuente de alimento. Durante los últimos 100 años, se han desarrollado y aplicado muchas estrategias de erradicación y supresión de *L. dispar.* Proyectos de investigación recientes incluyen varios enfoques moleculares, algunos de los cuales han dado como resultado importante información y técnicas útiles, algunas de las cuales están aun

en estado de desarrollo. Estos proyectos abordan un amplio rango de tópicos de investigación, incluyendo la determinación de las bases genéticas de la virulencia del virus de la poliedrosis nuclear (NPV) y la examinación de un mutante del NPV que reduce la producción en cultivos de células, y la detección de la cepa asiática de *L. dispar* y de los híbridos entre las dos cepas. Otro proyecto iniciado, tiene por objeto aislar el gen vitelogénico en *L. dispar*, a fin de examinar el papel de la ecdisona, posiblemente importante para diseñar un uso racional de pesticidas contra *L. dispar*. Un nuevo proyecto en camino hará posible desarrollar un método molecular para identificar y distinguir varios biotipos de microsporidios (Protozoa) patógenos de *L. dispar*. Estos esfuerzos en investigación fueron iniciados por el Servicio Forestal de los Estados Unidos de América, así como también por institutos, agencias y universidades norteamericanas y europeas que cooperan en los programas de control de la polilla gitana en América del Norte.

Studies using molecular techniques are important components of a cooperative biological control program targeting the gypsy moth, *Lymantria dispar*, in the United States. This presentation is a overview of several published projects and is intended as a review of the use of some molecular techniques to address specific questions within the gypsy moth program. The projects discussed in this report were conducted by US Forest Service scientists, as well as researchers at university and research institutions.

History of gypsy moth introduction. The gypsy moth, *Lymantria dispar* L., was accidentally introduced from Western Europe into northeastern North America in 1868-69. Within 20 years, this forest tree defoliator was in outbreak status due to the favorable temperate climate and the preponderance of *Quercus* species that dominated the northeastern forests and are a preferred food. *Lymantria dispar* was introduced without the compliment of natural enemies that occur in European populations, resulting in more frequent and severe outbreaks and higher population densities than those which occur in Europe (Leonard, 1981).

In addition to the introduction of *L. dispar* from Western Europe, an Asian strain of *L. dispar* has been periodically introduced. Unlike the European strain, the Asian *L. dispar* females, as well as a small percentage of females that are hybrids between the two strains, are capable of flight, increasing dispersal capability (Cardé, 1994). The Asian strain also utilizes more host plant species as an adequate food source for development, maturation and reproduction (Montgomery & Baranchikov, 1995).

Control efforts: During the past 100 years, many strategies for eradication and suppression of European *L. dispar* have been developed and applied. These initially included the burning of infested trees, trapping larvae, the use of acid and Paris green as insecticides, and a Federal domestic quarantine. Later, cryolite, DDT and carbaryl were used (McManus & McIntyre, 1981).

With increasing emphasis on reducing environmental pollution and the recognition that *L. dispar* will probably never be completely eradicated in North America, other control methods replaced the earlier efforts and an intensive research program was developed to find alternative controls. Dimilin, a chitin synthetase inhibitor, and *Bt kurstaki* became the insecticides of choice, especially for spot eradication, and the Forest Service developed "Gypcheck", a formulation of an *L. dispar* specific nuclear polyhedrosis virus (LdMNPV). Studies on the release of natural enemies were renewed (McManus & McIntyre, 1981). Several species of predators and parasitic Diptera and Hymenoptera have been released. Two pathogens, the naturally occurring NPV and the an Asian *L. dispar* entomophthoran fungus *Entomophaga maimaiga*, cause epizootics in *L. dispar* populations. The effects and interactions of these pathogens, as well as the microsporidia (Protozoa) which as yet are only found in European *L. dispar* populations, are currently under study in several laboratories in North America and Europe.

Molecular approaches to *L. dispar* research: Recent research projects include several molecular approaches, some of which have resulted in important information and usable techniques;

others are currently in developmental stages. These projects have addressed a wide range of research topics, including interruption of natural functions of *L. dispar*, genetically engineered natural enemies, detection of the Asian strain of *L. dispar* and Asian/European hybrids, detection of functional problems in natural enemies, and identification of natural enemies, particularly pathogens.

Interruption of natural functions: Hiremath & Lehtoma (1997) isolated, cloned, and studied the structure of the vitellogenin gene which produces vitellin, a precursor of the egg yolk protein, in *L. dispar*. The nucleotide sequence was found to be significantly different from Vg genes in other insects, including *Bombyx mori* , another lepidopteran. In other insect systems, the Vg gene is stimulated or unaffected by the presence of juvenile hormone (JH), but Vg is suppressed by JH in *L. dispar* (Hiremath & Jones, 1992). The role of JH may possibly be important in designing biorational *L. dispar* pesticides, and the Vg gene may be ideal for genetic control strategies such as incorporation of insecticidal genes (Hiremath & Lehtoma, 1994).

Genetically engineered natural enemies: D'Amico *et al.* (1995) genetically engineered the *Lymantria dispar* LdMNPV for non-persistence by removing the gene coding for the polyhedra, the environmentally-resistant protein matrix containing multiple virions. A galactosidase marker gene was inserted in its place. *Lymantria dispar* larvae, infected with the engineered virus, were released in the center of a test plot where larvae infected with the wild-type virus were also released. Eighteen million uninfected larvae were also released in the plot. Larvae were then collected weekly from the plot and reared individually in diet cups in the laboratory. Mortality from LdMNPV was recorded and the location of larvae turning blue (infected with engineered virus) showed how the virus spread in the gypsy moth population.

Distinguishing the Asian and European strains of *L. dispar*: As mentioned previously, the Asian gypsy moth is potentially a more harmful pest in North American forests than the established European strain (henceforth North American strain). Several spot introductions of the Asian strain have occurred on the East Coast, West Coast, and around Great Lakes shipping ports (Garner & Slavicek, 1996), probably due to the transport of egg masses on shipping containers. Eradication methods (primarily *Bt* sprays) appear to have been successful in containing the introductions but it is a difficult situation to assess because the Asian and European strains are nearly identical morphologically.

Garner & Slavicek (1996) identified a RAPD-PCR marker that distinguished between the two *L. dispar* strains and their hybrids. They tested 322 decanucleotide primers and found one that consistently amplified a 696 basepair fragment in the Asian strain of *L. dispar* and a 591 basepair fragment in the North American strain. The fragments were cloned and sequenced and found to be identical except for the absence of a 105 basepair fragment in the European strain and five nucleotides showing fixed differences between the strains.

Primers (17 and 20 bases) were selected from sequences flanking the insertion/deletion region and were used to analyze the DNA from the two *L. dispar* strains. Two primers amplified a 345 basepair fragment in the Asian strain and a 240 basepair fragment in the American strain. These fragments have been designated the FS-1 marker and have been shown to correctly identify the North American *L. dispar* strain (96% accuracy), the Asian strain (100% accuracy for Far East) and the hybrids (96% accuracy). The primers have been used to identify the genetic origin of male moths captured in pheromone traps.

Function of natural enemies: Bischoff & Slavicek (1997) discovered an *enhancin* gene for LdMNPV. *Enhancin* genes, previously found only in granulosis viruses, encode proteins that enhance the infection of other baculoviruses in a host. They appear to function by degrading the peritrophic membrane lining the host's midgut and by increasing the fusion of the virus nucleocapsids to the midgut cells (Derksen & Granados, 1988). During sequencing of a *Bam*HI/*Eco*RI fragment to which a mutant gene mapped in an LdMNPV isolate, Bischoff & Slavicek (1997) located an open reading frame with homology to the GV *enhancin* genes. The LdMNPV protein encoded by the *enhancin* gene is shorter than the GV proteins and shows an average 31% amino acid identity. The gene was disrupted with a *cat* gene insert to determine if the protein has an effect on the virus potency. Bioassays of

the *enhancin cat* virus showed that virulence was significantly decreased compared to an LdMNPV isolate with a nondisrupted *enhancin* gene, but killing speed was not affected. A genetically engineered strain of LdMNPV is currently being constructed that overexpresses the *enhancin* gene, with the goal of increasing the potency of the viral strain.

In addition to the above study, Slavicek *et al.* (1995) discovered that several isolates of LdMNPV lost the ability to form the protein matrix polyhedron during serial passage of the virus in tissue culture. These few polyhedral (FP) virus strains have few virions in the polyhedra that are produced and are less potent than strains which do not lose this function. The mutation is rapid, 90% of the virus present exhibits the mutant phenotype after three serial passages.

In order to test the hypothesis that several genes are involved in polyhedra formation, *L. dispar* cells (652Y) were coinfected with various combinations of two PF virus isolates (Bischoff *et al.*, 1994). The recombinations yielded some wild-type viruses, and marker rescue was performed for each combination of PF mutants using a cosmid library. Five genetic loci were identified that impacted polyhedra formation and virion occlusion. This project continues with the effort to find a strain of the LdMNVP that does not mutate in cell culture (Slavicek, 1997).

Identification of pathogens: A new project is underway in the laboratory of the author to develop RAPD-PCR markers to identify and distinguish between several biotypes of microsporidian (Protozoa) pathogens of *L. dispar.* Microsporidia found in different *L. dispar* populations in Central Europe are morphologically similar but show differences in pathogenicity and host range. Genetic markers could greatly assist efforts to identify these microsporidia for biological control programs and be used to study the evolution of microsporidia in isolated *L. dispar* populations.

This report is far from comprehensive; only final results of extensive work have been reported. Many other projects have been completed or are in progress. Nevertheless, the information presented here demonstrates the diversity of research efforts that have been undertaken in an effort to develop a rational, safe, and efficacious program for the biological control of a serious pest insect.

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